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Escherichia coli ß-galactosidase is heterogeneous with respect to a requirement for magnesium

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Abstract

Commercially obtained *E. coli* ß-galactosidase was stored at 25 °C in buffer containing 1 mM $MgCl_2$ and in buffer containing no added $MgCl_2$. Samples were removed at set times and the activity of individual enzyme molecules assayed. When stored in the presence of 1 mM magnesium, the number of active molecules did not change over a 2.5-h period. When stored in the absence of added $MgCl_2$, over half the enzyme molecules became inactive within the first hour. However, those molecules which retained activity remained active for the duration of the experiment. This indicates that there may exist two populations of *E. coli* ß-galactosidase, one which requires storage in the presence of the higher concentration of Mg^{2+} in order to remain active. There was no observed correlation between this requirement for magnesium and reaction rate. Additionally, the presence of the 1 mM $MgCl_2$ was found to decrease the average activity of the ß-galactosidase molecules under the conditions employed.

Introduction

The activity of individual molecules of lactate dehydrogenase (Xue & Yeung 1995), bovine (Craig *et al.* 1996, 1998) and *E. coli* (Polakowski *et al.* 2000) alkaline phosphatase, ß-galactosidase (Craig & Dovichi 1998), cholesterol oxidase (Lu *et al.* 1998) and horse raddish peroxidase (Edman *et al.* 1999) have been reported. In every case, the activities of the individual molecules of the given enzyme differed, in some by greater than one order of magnitude. This leads one to speculate that such microheterogeneity may be a general feature of enzyme molecules. This heterogeneity is not limited to activity. Activation energy of catalysis of alkaline phosphatase was found to vary amongst individual molecules (Craig *et al.* 1996). It is likely that such microheterogeneity extends to additional properties of enzyme molecules.

E. coli ß-galactosidase (EC 3.2.1.23) is a tetramer of identical 1023 amino acid monomers, each with a mass of 116 kDa. The enzyme contains 2 bound magnesium residues per monomer (Huber *et al.* 1994; Jacobson *et al.* 1994). The enzyme has been found to be activated by the presence of magnesium in the assay mixture (Martinez-Bilbao & Hubner 1994; Tenu *et al.* 1972). In fact, magnesium is typically included as a component of ß-galactosidase assay buffers for this reason. Here we report that *E. coli* ß-galactosidase is heterogeneous with respect to a requirement for magnesium in order to maintain activity during storage.

Materials and methods

Chemicals:

Genescan™ polymer was purchased from PE Applied Biosystems. A 1000 ppm magnesium stock was obtained from SCP Science. Sodium resorufin and resorufin-β-D-galactopyranoside were from Molecular Probes. All other chemicals were supplied by Sigma.

β-galactosidase standard assay:

The activity of stock *E. coli* ß-galactosidase (product number G-4155, Sigma) was measured by monitor-

ing the change in absorbance at 410 nm of a solution of 93 mM sodium phosphate (pH 7.3) containing 2.3 mM *o*-nitrophenyl β-D-galactoside, 1.0 mM magnesium chloride, 112 mM 2-mercaptoethanol and 0.02 – 0.1 units of enzyme. Incubation was at 37 °C. One unit of ß-galactosidase is defined as that amount which will hydrolyze 1.0 μmole of *o*-nitrophenyl ß-D-galactoside per minute under these conditions (Craven *et al.* 1965). The millimolar extinction coefficient for *o*-nitrophenol is 3.5 mM^{-1} cm^{-1}. Units were converted to concentration using a specific activity of 700 U mg^{-1} (Sigma product information) and a mass of 464 kDa.

Instrumentation:

The capillary electrophoresis laser-induced fluorescence instrument was assembled in-house at the University of Winnipeg. Details of the design have been published (Arriaga *et al.* 1995). Electrophoresis was conducted at 400 V/cm (injection end negative). Excitation was at 543.5 nm using a He Ne laser (Melles Griot). Excitation power was 900 μW, as measured with a LaserCheck^{TM} meter (Coherent). The sheath flow cuvette had an inner 250 μm $\times$ 250 μm bore. Emission was collected through a 580DF40 filter (Omega Optical). Photomultiplier tube (PMT) bias was 1100 V. Sheath and running buffers were 50 mM HEPES (pH 7.3) containing 0.0005% BSA and a 1/1000 dilution of the proprietary Genescan^{TM} polymer.

Capillary preparation:

A 10 μm internal diameter, 145 μm external diameter fused-silica capillary (Polymicro Technologies) was cut to a length of 40 cm and the polyimide coating removed from the detector end by flame. The capillary was placed in the instrument and flushed by pressure injection with 100 mM NaOH for 5 min. The capillary was coated by pressure injection with a 75% dilution of Genescan^{TM} polymer in 50 mM HEPES (pH 7.3) for 20 min. The capillary was then flushed with running buffer.

Single molecule ß-galactosidase assays:

The protocol is a modification on that published previously (Craig & Dovichi 1998). Stock solutions of ß-galactosidase obtained by the supplier were diluted 7 orders of magnitude in 50 mM HEPES containing 0.05% BSA. These stock 10^7 fold diluted enzyme solutions were stored at 25 °C.

In order to reduce the resorufin present as an impurity in the resorufin-ß-D-galactopyranoside, a 1 mM solution of the substrate in 5 mM HEPES (pH unadjusted and approximately 5) was extracted with an equal volume of $CHCl_3$ three times immediately prior to use.

At set times after the initial 10^7 fold dilution of the enzyme, substrate, buffer and an aliquot of the diluted stock enzyme solutions were mixed to produce a sample containing 50 mM HEPES (pH 7.3), 200 μM resorufin-ß-D-galactopyranoside, 10^9 fold dilution of ß-galactosidase, 1000 fold dilution of Genescan^{TM} and 0.0005% BSA. The capillary was flushed with the sample by pressure injection for 3 min followed by incubation at 25 °C for 15 min. Following the incubation, the capillary was subjected to electrophoresis. Blanks contained no enzyme.

In order to denature any contaminating enzyme, all solutions, tips and vessels were autoclaved prior to use, with the exception of the stock BSA which was prepared in 100 mM HCl.

In those samples that were stored and assayed in the presence of added magnesium, the storage, assay, sheath and running buffers were prepared as above with the exception that they also included 1 mM $MgCl_2$. In all samples the enzyme dilution used was identical.

Resorufin standards:

4×10^{-8} M sodium resorufin in running buffer was injected into the capillary electrokinetically for 5 s at an electric field of 125 V cm^{-1} and subjected to electrophoresis.

Magnesium analysis:

Analysis of the 'magnesium free' storage buffer for magnesium was carried out using a Perkin-Elmer flame atomic absorption spectrometer. An air acetylene flame was used with a Perkin-Elmer hollow cathode lamp. The monochrometer was set at 285.2 nm with a slit width of 0.7 nm. Standards were made from 1000 ppm magnesium stock by serial dilution using deionized (18 MΩ) water.

Results and discussion

Capillary electrophoresis has been used in the assaying of single enzyme molecules (Xue & Yeung 1996; Craig *et al.* 1996, 1998; Craig & Dovichi 1998; Polakowski *et al.* 2000). A capillary is filled with a very dilute solution of enzyme containing a fluorogenic substrate. Enzyme concentration is such that only a few enzyme molecules will be present within the capillary volume. During an incubation period the enzyme molecules convert the substrate into a fluorescent product. Due to a limited time for diffusion, the product molecules remain in the immediate vicinity of the enzyme molecule which produced them. This results in the formation of localized pools of product surrounding the enzyme molecules. Electrophoretic migration of these product pools past a highly sensitive fluorescence detector results in the formation of peaks, each representing the activity of a single molecule. In the assay used in this study, ß-galactosidase catalyzes the hydrolysis of the galactosidase moiety from the substrate resorufin-ß-D-galactopyranoside, resulting in the formation of the fluorescent dye resorufin.

Fused silica capillaries have a negative inner surface charge due to deprotonation of surface silanol groups. One effect of this charge is a net flow of buffer from the cathode to the anode in the presence of an electric field. This is referred to as electroosmotic flow (EOF). The mobility of an analyte in the capillary is the sum of the EOF and the analyte's electrophoretic mobility, which is approximated by the charge/mass ratio and is directed toward the electrode of opposite charge. In an untreated capillary the EOF is typically larger than the electrophoretic mobility of a given analyte. This results in all analyte, regardless of charge, eventually migrating from the cathode to the anode. The Genescan^{TM} polymer adheres in a thin layer to the surface of the capillary resulting in a large drop in the surface negative charge. This in turn greatly reduces the EOF. It does not, however, affect the electrophoretic mobility of the analyte. Thus in a coated capillary, electrophoretic mobility of many analytes exceeds the EOF and in these cases net mobility of anions will be towards the cathode.

At pH 7.3, the substrate resorufin-ß-D-galactopy ranoside has no net charge and the product, resorufin, has a net negative charge. Although the substrate is much less fluorescent than the product, at a concentration of 200 μM it produces sufficient signal to saturate the PMT. In this protocol we use a capillary that has been coated with Genescan^{TM} and place the detector at the cathode end of the capillary. The substrate, being neutral, will have no electrophoretic mobility and will migrate with the residual EOF and away from the detector, thus producing no signal. The product, having a negative charge, will have an electrophoretic mobility towards the detector which opposes the EOF. Since the capillary is coated, the electrophoretic mobility of the product exceeds the EOF and net migration is towards the detector, allowing product to produce signal. Thus the use of a coated capillary allows one to ensure that the residual substrate produces no signal that will interfere with that from the product.

Standard assay of the commercially obtained solution of ß-galactosidase determined the concentration of the active enzyme to be nominally 1.8 μM.

ß-galactosidase was stored and assayed in the presence and absence of 1 mM Mg^{2+}. Analysis of the 'magnesium free' storage buffer using atomic absorbance spectrometry determined the concentration of Mg^{2+} to be 49 μM. This magnesium was presumably present as an impurity in the components used to make the solution, likely the NaOH used to adjust the pH.

At set times after the initial 10^7 fold dilution and storage at 25 °C, the samples were assayed. The sample stored in the buffer containing 1 mM $MgCl_2$ was assayed starting 16, 60, 115 and 159 min after the initial dilution. The sample stored in the buffer containing no added Mg^{2+} was assayed starting 12, 54, 98 and 147 min after the initial dilution. The sample stored in the presence of 1 mM $MgCl_2$ was assayed in the presence of 1 mM $MgCl_2$. The sample stored in the absence of added Mg^{2+} was assayed in the absence of added Mg^{2+}.

Figure 1 shows the electropherogram resulting from the assay of the sample stored in 1 mM $MgCl_2$ and assayed at 16 min after the initial 10^7 fold dilution (top trace). The bottom trace is that of a blank. Thermodynamically favorable reactions proceed even in the absence of enzymes to catalyze them, albeit at a much slower rate. The plateaus formed in each trace are due to product formed from the non-enzymatic hydrolysis as well as residual product present in the substrate after the 3 extractions. When enzyme is present, additional product is formed which is seen as a peak sitting atop this plateau. Each peak represents the activity of an individual enzyme molecule. In the upper trace, 26 peaks are clearly discernable. Expansion of the peaks centering at 145 and 535 s reveal that both these peaks result from sets of 2 incompletely resolved peaks. Thus the total number of

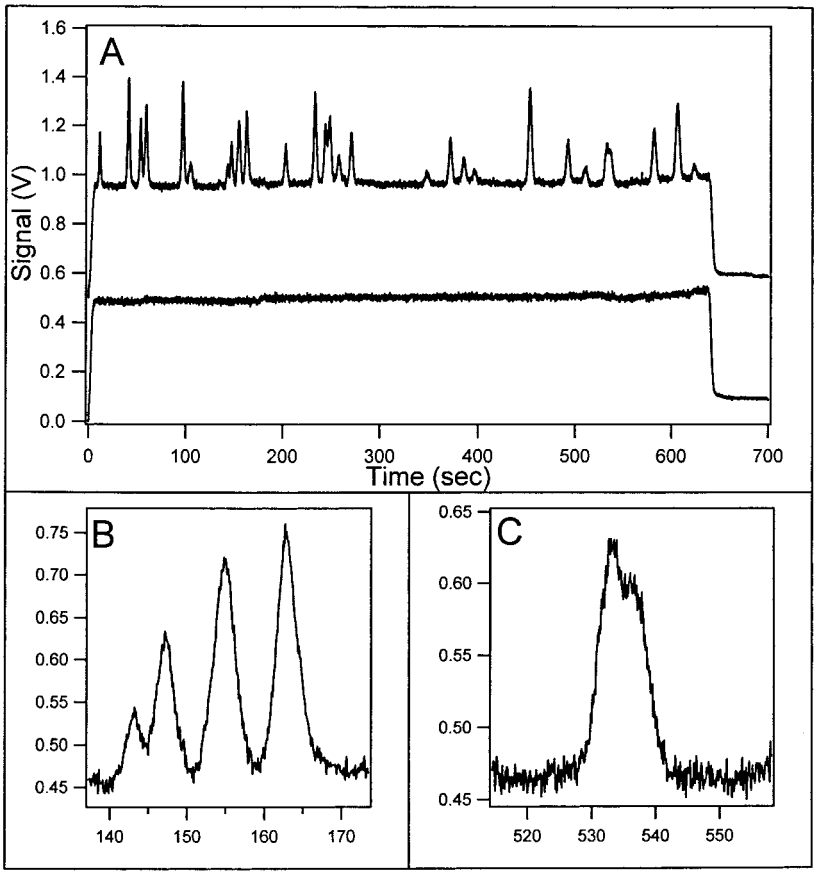

Fig. 1. The resultant electropherogram from the incubation of 1.8 fM ß-galactosidase initiated after storage for 16 min is shown. Storage was at a concentration of 180 fM enzyme in 50 mM HEPES (pH 7.3) containing 1 mM $MgCl_2$ and 0.05% BSA at 25 °C. The sample was incubated 15 min in 50 mM HEPES (pH 7.3) containing 200 μM resorufin-ß-D-galactopyranoside, 1 mM $MgCl_2$, 0.0005% BSA and 1/1000 dilution of Genescan^{TM}. The sample was then subjected to electrophoresis at an electric field of 400 Vcm^{-1}, injection end negative (A, upper trace). The blank contained no enzyme (A, lower trace). B and C are expansions of the 140–170 and the 520–550 s regions respectively of the upper trace of A.

peaks in this trace is 28. In the blank trace, no peaks (3σ) are detected.

A 40 cm long 10 μm internal diameter capillary has an inner volume of 31 nL. When filled with a 1.8 fM solution of enzyme one would expect there to be, on average, 56 ymoles (1 ymole $= 10^{-24}$ moles) or 33.6 molecules of enzyme present. However, the number of molecules taken up by random sampling from solution is dictated by the Poisson distribution. That is, the standard deviation of the mean number of molecules taken must be, within error, the square root of the mean. Additionally, the position of the molecules within the capillary must be random. Based on the enzyme concentration, as approximated by the standard assay, one would expect 33.6 ± 5.8 enzyme molecules per capillary volume. Thus one would predict approximately 33.6 ± 5.8 randomly positioned peaks to be detected per run. In the $t = 16$, 60, 115 and 159 min incubations we observe 28, 30, 18 and 24 peaks, respectively. These 4 runs produce a Poission dominated distribution of 25.0 ± 5.3 peaks per run.

Assays of the sample stored in the buffer containing no added Mg^{2+} performed at $t = 12$, 54, 98 and 147 min yielded electropherograms containing 36, 12, 9 and 16 peaks, respectively. The 4 runs together do not form a Poisson dominated distribution but rather yield a mean of 18.3 ± 12.2. The standard deviation is almost three times that of a Poission distribution with a mean of 18.3. This is due to the first run producing three times the number of peaks as the average of the following three. However, the three latter runs are Poisson distributed with a mean of 12.3 ± 3.5. The square root of 12.3 is 3.5. Thus this set of runs is represented by an initial run with a peak count which

is more consistent with that observed from the sample stored in 1 mM $MgCl_2$ and three latter runs that fall within one distribution. This distribution has a mean that is less than half that produced by the sample stored in 1 mM $MgCl_2$.

This data indicates that when stored at 25 °C in 50 mM HEPES (pH 7.3) containing 1 mM $MgCl_2$and 0.05% BSA, ß-galactosidase remains active for at least 2.5 h. However, when stored in absence of added Mg^{2+}, the concentration of active enzyme is reduced by less than half within 1 h. The fraction of enzyme that remains active after one hour remained active for the duration of the runs. Therefore it would appear that there are at least 2 populations of ß-galactosidase present with respect to a requirement for Mg^{2+}. One population remains active in the presence of both 1 mM and 49 μM Mg^{2+} and the other population retains its activity only when stored in the presence of the higher concentration of magnesium.

All of the runs performed produced electropherograms that were very similar to that of Figure 1, varying only with respect to the number of peaks and their exact position. This is of course guaranteed within a series of identical samples by the random sampling process. It should be noted that the least active enzyme molecules produced peaks that were just above the limit of detection (3σ). It is therefore possible that some enzyme molecules may have been present which produced peaks that were not detected. The average number of peaks observed per run may therefore be an underestimate of the actual number of enzyme molecules present.

The calculation of the number of enzyme molecules expected in the capillary volume is dependent upon the value used for the specific activity. Specific activities are notoriously unreliable. They are determined upon purification of the enzyme and measurement of the protein concentration. This assumes that all of the protein in the sample is active and all of it is the protein of interest. Additionally, protein assays are based on comparison of signal obtained from the binding of a dye to a solution of the protein of interest and to a solution of an easily obtained protein, typically BSA, being used as the primary standard. It is generally recognized that some variation in response is obtained from protein-to-protein upon binding of a particular dye. Thus calculations of enzyme concentration based on specific activity is subject to a relatively large degree of uncertainty.

Since the standard deviation in a Poisson distribution is the square root of the mean, the relative error due to sampling decreases as the average number of molecules captured increases. One might therefore expect it to have been advantageous to have assayed the samples at a lesser dilution. This would have made differences in the observed number of peaks in the two samples more apparent. However, as more enzyme molecules are present in the capillary, clustering of peaks in the electropherogram increases. This makes it difficult to determine with a reasonable degree of certainty the number of peaks and their areas. This is due to the increased risk of two enzyme molecules forming product pools that overlap in the electropherogram to such an extent that they appear to be one peak. Regardless, assays performed at higher concentrations showed the same effect of storage in the presence of no added and 1 mM magnesium (data not shown). However, accurate measurement of the peak numbers and areas were not possible due to a large degree of peak overlap.

Peak width increases with retention time. Early eluting peaks were produced by enzyme molecules that were located near the detection end of the capillary. They therefore have a shorter distance to travel in the capillary than the later eluting peaks which were formed by enzyme molecules further down. The shorter distance to travel results in decreased band broadening. Since peak widths are not homogenous, quantitation requires measurements of peak area. Using the program PeakFit^{TM} (SPSS Science), peaks were fitted to one Gaussian peak and integrated. In the cases where two or more peaks were not completely resolved, the region of the curve was fitted to two or more Gaussian peaks. In order to avoid a loss in resolution, data was not smoothed. Peak areas were compared to resorufin standards and enzyme turnover numbers were calculated.

Figure 2 is a histogram of the enzyme turnover numbers of the samples that were stored in 'magnesium free' buffer prior to assay. There is no obvious difference, with respect to the distribution of activities, between the sample assayed at $t = 16$ min, which contains both populations of the enzyme, and those assayed at $t = 60$, 115 and 159 min, which contain the population that remains active when stored at the lower Mg^{2+} concentration. Average turnover numbers of these two groups were $22\,000 \pm 14\,000$ reactions/min and $17\,000 \pm 12\,000$ reactions/min, respectively. This indicates that whatever the structural feature that distinguishes these two populations is, it does not appear to be a dominant factor determining the heterogeneity of the enzyme activity.

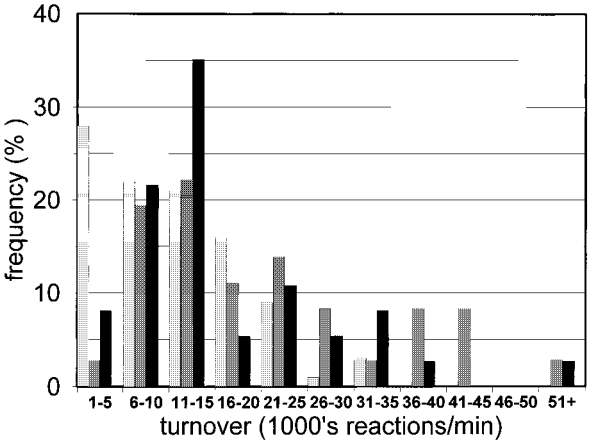

Fig. 2. A histogram of the composite activity distribution from all 4 runs performed on the ß-galactosidase molecules stored and assayed in 1 mM $MgCl_2$ (light shade) is shown. The activities of the $t = 12$ min run (medium shade) and a composite of the activities from the $t = 54$, 98 and 147 min runs (dark shade) of the sample stored and assayed in buffer containing no added Mg^{2+} are also shown.

Comparison of the distribution of the samples stored and assayed in the presence of 1 mM and in the 'magnesium free' buffer show that at the high magnesium concentration the enzyme activity distribution is shifted towards a lower activity (Figure 2). The average observed rate was $11\,000 \pm 7000$ reactions/min. This indicates that the higher magnesium concentration employed inhibited the enzyme. Thus, under the conditions employed, magnesium has the effect of stabilizing yet inhibiting ß-galactosidase.

It is difficult to speculate on the structural causes for the observed heterogeneity of ß-galactosidase with respect to its requirement for exogeneous magnesium to retain activity. Subfractionation of the heterogeneous populations into homogeneity followed by structure determination would be the preferred, albeit time consuming, process to undertake.

Xue & Yeung (1996) propose that the observed differences in the properties of individual lactate dehydrogenase molecules arises from different conformations that are stable over the timeframe of many minutes. Experimental evidence indicates however that enzyme heterogeneity must result from, at least in part, by covalent differences between individual molecules (Dovichi *et al.* 2000). Firstly, commercially obtained *E. coli* alkaline phosphatase can be fractionated by isoelectric focusing into three homogeneous subpopulations with respect to individual molecule activities. Storage in the absence of protease inhibitors results in the isolation of at least 12 active subpopulations, implying that one source of heterogeneity is partial proteolysis. Populations of bovine alkaline phosphatase, ß-galactosidase, lactate dehydrogenase and cholesterol oxidase, shown to be microheterogeneous with respect to the activities of individual molecules, can be shown by iso-electric focusing to be heterogeneous (Polakowski *et al.* 2000). Due to alterations of the pK_a's of protein functional groups by local environments it is theoretically possible to separate protein conformers by iso-electric focusing although it is unlikely that this alone can account for the observed data.

If multiple conformations, stable over the timeframe of several minutes and each with a different activity, were possible for a given enzyme, one might predict that partial thermal denaturation of a population would result from the driving of the population into successively less active conformers by the increase in temperature. However, studies with bovine alkaline phosphatase showed that thermal denaturation is catastrophic. Loss of activity occurs through the loss of the number of active molecules and not through a decrease in the activity of the individual molecules (Craig *et al.* 1996; Dovichi *et al.* 2000).

Finally, most of the studies on single enzyme molecules to date have relied on commercial preparations of enzymes. It is possible that the purification process results in the generation of microheterogeneity as an artifact. *E. coli* cells have been ruptured by one cycle of freezing and thawing in the presence of protease inhibitor cocktail (without metal chelators) in the presence of buffer at pH 7.3, 1 mM $MgCl_2$ and 50% glycerol. Single ß-galactosidase molecule assays of the resultant sample yielded a population of heterogeneous activities (Craig & Hall, unpublished). This indicates that despite a very mild treatment, the population is found to be equally microheterogeneous to the commercial preparation. The observed heterogeneity does not appear to be an artifact of the invasive commercial purification process.

It is premature to speculate on how any structural differences between individual ß-galactosidase molecules might account for the different dependencies on exogenous magnesium. Since magnesium comprises part of the enzyme's active site one might at first speculate that the loss in activity somehow reflects a loss of these magnesium ions. However, we find this dependency to occur when comparing the relatively high concentrations of 49 μM and 1 mM. It is possible that this dependency might reflect stabilization of

some structure within the enzyme molecule other than the catalytic center. If the observed inactivation at the lower concentrations of magnesium were due to a loss of the ion from the catalytic center this would indicate that the catalytic sites of the two observed populations would differ. If this was the case one might expect to have found discernable differences in the activities of the two populations.

Enzyme studies typically involve measurements on large ensembles of molecules. These experiments can determine the effect of a treatment on overall activity of the enzyme population. However, assaying of individual molecules allows one to determine the actual fraction of molecules that remain active, what this activity is and any effect on the distribution of activities. In this study we have shown that storage of ß-galactosidase in 1 mM magnesium results in the maintenance of the number of active molecules. Storage in the presence of 49 μM magnesium causes an initial decrease in the number of active enzyme molecules. Those molecules that remained active retained their activities for the duration of the experiment. This demonstrates that there may exist at least two populations of ß-galactosidase molecules with respect to stability. One population is not stable in the presence of 49 μM magnesium and the other is. Additionally, although the 1 mM magnesium has the effect of stabilizing the enzyme, it has an inhibitory effect on the catalytic rate under the conditions used.

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